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FINAL REPORT

A STUDY OF TRYPSIN INHIBITOR LEVELS AND THE EFFECTS OF EXCESS WHEY PROTEIN IN IMMUNOGLOBULIN ABSORPTION FROM DRIED BOVINE COLOSTRUM

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Final Report to the Saskatchewan Agriculture Development Fund for the Study Titled :

"A Study of Trypsin Inhibitor Levels and the Effects of Excess Whey Protein on Immunoglobulin Absorption from Dried Bovine Colostrum"

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Abstract/Summary

This report describes the assessment of immunoglobulins and trypsin inhibitor levels in four commercially available colostrum supplements for calves. The "active ingredients" in colostrum are the immunoglobulins which are absorbed into the bloodstream of the newborn calf to provide disease protection in the first weeks of life. Assessment of the total immunoglobulin levels demonstrated that the supplements contain 35-60 grams of immunoglobulin/dose which was similar to the levels claimed by the various manufacturers. The percentage of the total dry matter/dose which is comprised by immunoglobulin varies from 8 to 27%. To determine if the % immunoglobulin affects the uptake of the immunoglobulin into the bloodstream of the calf, newborn calves were fed 2 litres of colostrum containing 120-128 gms of immunoglobulin which was either 27% or 15% immunoglobulin. Calves fed the colostrum containing 27% immunoglobulin had higher serum immunoglobulins and higher absorption rates than calves fed colostrum containing 15% immunoglobulin.

Trypsin inhibitors (TI) are present at high levels in colostrum and act to prevent proteolytic digestion of immunoglobulins prior to absorption. Measurement of TI in these colostrum supplements demonstrated TI levels similar to fresh colostrum in the 2 supplements made from colostrum, while a supplement made from cheese whey had much lower levels and the supplement derived from serum very high levels (similar to those found in serum). The low levels in the cheese whey derived product may contribute to the apparent poor absorption of this product.

Fifty calves born at a local commercial dairy were randomized to receive either maternal colostrum or one of 4 colostrum supplements, the calves fed the maternal colostrum had significantly higher serum immunoglobulin levels than those fed any of the supplements and only those fed the colostrum-derived supplement with 27% immunoglobulin had levels over 10 g/L, the levels normally considered consistent with neonatal calf health.

These studies have shown that calves fed 3/4 colostrum supplements had inadequate levels of serum immunoglobulins. These 3 colostrum supplements had lower immunoglobulin mass, and lower percentage immunoglobulin. The % immunoglobulin as well as the mass of immunoglobulin in the product was shown to impact upon effective absorption of the immunoglobulins by calves. The TI levels were shown to be low in one product, which may also affect the levels of immunoglobulin achieved in the calves fed that product. These studies suggest that colostrum supplements should be colostrum-derived and confirms that they should have immunoglobulin mass in excess of 60 g/L and percentage immunoglobulin over 25%.

Executive summary

Newborn calves lack serum antibodies necessary for protection against the infectious disease agents in their environment. The newborn calf relies upon receiving antibodies (immunoglobulins) from the colostrum of the mother for health in the first weeks of life. The colostrum is the milk produced by the cow in the first days following parturition. In the days immediately prior to parturition, large quantities of immunoglobulins from the cows' serum concentrate in the colostrum. While in adult animals intact immunoglobulin molecules do not cross the cells of the gastrointestinal tract, in the newborn calf these cells are "open" for about 24 hours and allow the absorption of large macro-molecules, such as immunoglobulin, directly into the bloodstream.

Inadequate levels of colostral immunoglobulins are documented to occur in over 25 % of calves in even well-managed beef and dairy operations. Low levels of serum immunoglobulins in the first weeks of a life is a syndrome termed "failure of passive transfer of maternal immunoglobulin" (FPT). Failure of passive transfer is a well recognized cause of calf losses from bacterial and viral infections resulting in septicemia, pneumonia and gastrointestinal diseases.

There are a variety of management strategies designed to attempt to increase the level of colostral immunoglobulins in newborns, one of which is the feeding of commercially available colostrum supplements to high risk calves. In the past decade a number of products have been developed for use in preventing FPT in calves however most have been of dubious efficacy. Earlier research in our laboratory showed that some of the products have only low immunoglobulin levels which would not be of significant benefit if fed to newborn calves. In addition, recent studies from the USA have shown that feeding calves some products, with manufacturers claims of significant immunoglobulin mass, resulted in only low serum antibody levels consistent with FPT due to an apparent problem with absorption of the immunoglobulins from the products.

The factors which affect the absorption of immunoglobulin from "natural" maternal colostrum have been well studied. Most studies show that the critical factors are the time of intake (since the cells of the intestine can absorb the immunoglobulins into the bloodstream for only a short time period), the volume of colostrum that the calf consumes and the mass of the immunoglobulins in that colostrum. There is however, a complex interaction among these variables, for instance, feeding a given mass of immunoglobulins in a smaller volume results in better absorption (and higher immunoglobulin levels in the serum of the calf) than that same mass in a larger volume.

There are few studies that have examined the factors affecting the absorption of immunoglobulins from commercially prepared colostrum supplements, most of which are spray-dried products, however it has been demonstrated that some products have a lower than expected absorption of immunoglobulins given the mass of immunoglobulin and volumes of colostrum consumed. One factor which may impact on the amount of the immunoglobulin absorbed is the presence of other nonimmunoglobulin (whey) proteins in the colostrum. The pathway for absorption of macromolecules into the serum through the intestinal epithelial cells has a finite capacity and is a largely a nonselective process. Therefore, if there are high levels of nonimmunoglobulin proteins compared to immunoglobulins present in the colostrum, these molecules may compete with the immunoglobulins during the absorptive process and diminish their absorption.

One of the goals of this study is to measure the levels of immunoglobulins in commercially available colostrum supplements and determine the relative percentages of immunoglobulin to nonimmunoglobulin whey proteins. The results of this work demonstrated that the levels in most of the commercially available products are much lower than the levels found in good quality maternal colostrum (which is in excess of 25% immunoglobulin). The percentage of immunoglobulins in 3 of the 4 tested products; Colostrx^{R}, Immustart^{R} and Lifeline^{R}, are less than 10% based both upon the calculated levels (based on manufacturers claim) and those measured in this study. Only the product HEAD*START*^{R} has in excess of 25 % immunoglobulin.

An additional goal of the study was to experimentally determine if the addition of excess whey protein to spray dried colostrum would impact on its absorption. Newborn calves (n=10/group) were fed 2 doses of either unadulterated HEAD*START*[R] or HEAD*START*[R] to which 225 grams of excess whey protein was added to each dose. The addition of excess whey protein decreased the levels of immunoglobulin from 27% to 13% and resulted in a decrease in the serum immunoglobulin levels in calves from an average of 12 g/L to 8 g/L, a decrease of about 25%. The % apparent absorption of the immunoglobulin diminished from 31% in the unadulterated product to 19% in that to which the excess whey protein was added. These findings support the hypothesis that low percentage immunoglobulin may be partially responsible for low serum immunoglobulin levels achieved in calves fed products containing low less than 25% immunoglobulin.

Another factor which may influence the absorption of immunoglobulin molecules into the serum of the calf is the amount of trypsin inhibitor in the colostrum. Normal fresh colostrum contains high levels of this enzyme inhibitor which acts to prevent the normal digestive enzymes of the gastrointestinal tract from degrading the immunoglobulins. If colostrum supplements contain low levels of trypsin inhibitors compared to normal colostrum this may result in the immunoglobulins being degraded prior to their absorption into the serum. A goal of this study was to measure the levels found in some commercially available calf colostrum supplements and the levels in fresh, frozen and spray dried colostrum to determine if freezing, or the heat treatment involved in spray drying diminished trypsin inhibitor. It was shown that bovine colostrum has high levels of trypsin inhibitors which are not significantly affected by either freezing or spray drying. Commercially available colostrum supplements produced from bovine colostrum (HEAD*START*[R] and Immustart[R]) have levels of trypsin inhibitors similar to those found in fresh colostrum. A colostrum supplement produced from cheese whey (Colostrx[R]) has low levels of trypsin inhibitors similar to those found in whole milk. An immunoglobulin supplement manufactured from serum (Lifeline[R]) has high levels of trypsin inhibitors similar to those found in normal serum.

The final goal of this study was to examine the levels of immunoglobulins attained in the serum of newborn calves fed 2 doses of one of the four commercially available colostrum supplements or maternal colostrum. The results of these studies showed that the serum immunoglobulin levels attained in calves ranged from mean values of 6.9 g/L in the group of calves fed the product Colostrx[R] to 16.1 g/L in calves fed HEAD*START*[R] to levels of 24 g/L in those fed maternal colostrum. Among the supplement-fed calves, only the HEAD*START*-fed calves averaged serum immunoglobulin levels in excess of 10 g/L; the minimum levels considered desirable for calf health. The calves fed most colostrum supplements had significant increase in numbers of days sick/treatments in the first days of life compared to those fed either maternal colostrum or HEAD*START*[R].

Technical Report

1. The first aspect of this study was a comparison of the mass and percentages of immunoglobulins in four commercially available colostrum supplements: Colostrx[R] (Protein Technology Inc., Sanata Rosa, CA), Immustart[R] (Imu-Tek Animal Health, Fort Collins, CO), Lifeline[R] (American Protein Corp., Ames, IO) and HEAD*START*[R] (Saskatoon Colostrum Co. Ltd., Saskatoon, SK). Colostrx[R] is a byproduct of cheese manufacture, Lifeline[R] is derived from slaughterhouse blood and ImmuStart[R] and HEAD*START*[R] from excess dairy colostrum. The samples of Lifeline[R], Colostrx[R], and Immustart[R] were purchased from retail outlets, samples of HEAD*START*[R] were chosen randomly from the licensing serial for this product (serial 97-02).

The percentages of immunoglobulin in commercially available colostrum supplements; calculated from manufacturers claims are presented in Table 1.

Table 1. The average dry matter/dose, immunoglobulin/dose and calculated percentages of immunoglobulin in 4 commercially available calf colostrum supplements based upon manufacturers claims.

Supplement	Dry matter/dose (g)	Ig/dose (g)	% Ig
Colostrx[R] serial M9009	454	35	8
Immustart[R] serial 0369	400	35	9
Lifeline[R] serial E06880718	454	45	10
Headstart[R] serial 9702	225	60	27

These data show that the both the reported mass of immunoglobulin and percentage of immunoglobulins vary widely among commercially available calf colostrum supplements. Most recommendations for minimum quality of colostrum (mass of Ig/L) is 50 gms which suggests that 3/4 of these products do not contain adequate mass of immunoglobulins. Good quality maternal colostrum is in excess of 25% immunoglobulin, in contrast 3/4 products have significantly lower Ig levels.

2. The second goal of the study was the direct measurement of the levels of immunoglobulins in these colostrum products to compare measured levels with the manufacturers claims. The immunoglobulins IgG, IgM and IgA were measured using radial immunodiffusion methods (see appendix 1 for technical details of the procedures). The means levels measured and total immunoglobulin levels calculated from these measurements are presented in Table 2.

Table 2 Results of radial immunodiffusion analysis of levels of IgG

Supplement	IgG (g/dose)
Colostrx[R]	36.6
Immustart[R]	43.7
Lifeline[R]	57.1
Headstart[R]	60

The results of these studies showed that the measured levels of immunoglobulin G in all products were similar to or greater than the levels claimed by the manufacturers.

The measured percentages of immunoglobulin were calculated using the measured immunoglobulin levels and the total dry matter in each product. The results are presented in Table 3.

Table 3 The measured immunoglobulins dose and calculated percentages of immunoglobulin in 4 commercially available colostrum supplements.

Supplement	Dry matter/dose (g)	Ig/dose (g)	% Ig
Colostrx[R]	454	37	8.1
Immustart[R]	400	44	11
Lifeline[R]	454	57	12.5
Headstart[R]	225	60	27

This data shows that while the measured mass of immunoglobulin is similar to or higher than that claimed by the manufacturer, in 3/4 products, due to the high dry matter/dose of non immunoglobulin protein, the % Ig/dose is low.

4. One explanation for the reported low levels of immunoglobulins absorbed by calves fed commercial colostrum supplements is the possibility that a low percentage of immunoglobulin, compared to other proteins, would interfere with immunoglobulin absorption.

To investigate this hypothesis calves were randomized to receive 2 doses of either unadulterated HEAD*START*[R] (prepared according to package recommendations) or 2 doses of the same serial of HEAD*START*[R] to which 225 gms of whey protein/dose had been added. Calves were blood sampled prior to feeding and 24 hours after the second feeding; calves with detectable pre-feeding immunoglobulins were excluded from the analysis as these calves can be assumed to have suckled their dam. The results of this study are presented in Table 4.

Table 4 Comparison of immunoglobulin levels in calves fed 2 doses of either HEAD*START*[R] or HEAD*START*[R] plus excess whey protein

	HEAD*START*[R]	HEAD*START*[R] & Whey
Amount of dry matter/dose (g/dose)	225	450
Total dry matter fed (g)	450	1000
Immunoglobulin/dose (g/dose)	60	68*
Total Immunoglobulin fed (g)	120	132
% Immunoglobulin	27	15
Calf Serum Immunoglobulin (g/L) (IgG, M & A), n=10/gp	12	8
% Apparent Absorption**	31	19

* The whey protein contained 8 gm of immunoglobulin in 225 g dry matter
** % Apparent absorption is calculated as follows: average serum immunoglobulins (g/L) x 7% of body weight (kgms)/mass of Ig consumed (gms)

This experiment showed that the addition of 225 gm of additional whey protein (containing 3% immunoglobulin) to HEAD*START*[R] colostrum, which had the effect of decreasing the levels of immunoglobulin from 27% to 15%, decreased the absorption of the immunoglobulin into the bloodstream of the calf. The average serum immunoglobulin levels decreased from 12 g/L to 8 g/L; the calculated apparent absorption of the immunoglobulins decreased from 31% to 19%, a decrease of almost 40%.

The conclusion from this aspect of the study is that the absorption of immunoglobulins from colostrum supplements is related to the percentage of immunoglobulins as well as the total immunoglobulin mass consumed. Many previous studies have documented that calves should obtain a minimum serum immunoglobulin level of 10 g/L for normal neonatal health, this study shows that in order to achieve that level of serum antibody, even with consumption of over 130 gms of immunoglobulin mass, calves need to consume colostrum with immunoglobulin percentage greater than 15%.

5. Trypsin inhibitors are proteins which prevent the gastrointestinal enzyme, trypsin, from degradation of the immunoglobulins within the gastrointestinal tract. Colostrum normally contains high levels of this protein which enables the immunoglobulins to remain intact for absorption into the bloodstream of the neonate. One reason immunoglobulins may be poorly absorbed from colostrum supplements may be low levels of this enzyme inhibitor or loss of its activity during the drying process. This aspect of the study was designed to examine the levels of trypsin inhibitors in fresh, frozen and spray dried colostrum, in milk, serum and in reconstituted samples of the 4 dried calf colostrum supplements. The technical details of the assay are presented in Appendix 2 and the results in Table 4.

Table 4 Average levels of trypsin inhibitors in commercially available colostrum supplements and in fresh and frozen colostrum, skim milk and normal serum

Colostrum or Supplement	Trypsin Inhibitor (mg trypsin inhibited/mL of colostrum or supplement)
Fresh Colostrum*	0.247
Frozen Colostrum**	0.247
Pooled Colostrum# 1	0.260
Pooled Colostrum #2	0.253
Spray dried Pool #1	0.233
Spray dried Pool #2	0.245
2% Milk	none detected
Bovine Serum	0.353
Colostrx®	0.099
Immustart®	0.258
Headstart®	0.252
Lifeline®	0.366

* Randomly selected first milk colostrum from a mature dairy cow from the University of Saskatchewan Dairy Barn.

**Colostrum from the mature cow was frozen in a -20 degree C freezer for 7 days, thawed and tested immediately.

This study shows that fresh bovine colostrum has high levels of trypsin inhibitors which are not significantly affected by either freezing or spray drying. Commercially available colostrum supplements produced from bovine colostrum (HEAD*START*[R] and Immustart[R]) have levels of trypsin inhibitors similar to those found in fresh colostrum. A colostrum supplement produced from cheese whey (Colostrx[R]) has low levels of trypsin inhibitors similar to those found in whole milk. Immunoglobulin supplements manufactured from serum (Lifeline[R]) has high levels of trypsin inhibitors similar to those found in normal serum

The low levels of trypsin inhibitors found in Colostrx[R] may contribute to poor absorption of immunoglobulin molecules when this product is fed, the higher levels found in the colostrum and serum derived products suggest that this factor does not contribute to poor absorption in those products.

5. The final goal of this study is to measure the levels of immunoglobulins attained in the serum of newborn calves fed four commercially available colostrum supplements. Fifty newborn calves were randomized to receive 2 doses of either Colostrx[R], Immustart[R], HEAD*START*[R], Lifeline[R] or maternal colostrum. Calves were fed the first dose as soon as observed after calving and the second dose 6-8 hours later. Blood samples were collected by jugular puncture prior to feeding and 24 hours after the second feeding. Calves are excluded if the first dose of supplement was not fed within 6 hours of birth and the second dose within 6-8 hours of the first. Calves are also excluded if there is evidence for immunoglobulin in the prefeeding blood sample suggesting that the calf has been suckled by the cow. Table 5 shows the ranges of serum immunoglobulins (IgG) measured in these calves and Table 6 shows the calculation of the apparent absorption of the immunoglobulin mass fed.

Table 5. Levels of serum immunoglobulins in newborn calves 24 hours after feeding 2 doses of maternal colostrum or a commercially available colostrum supplement.

Colostrum Source	Range Serum IgG (g/L)	Mean Serum IgG (g/L)
Maternal	11-36	24
Colostrx[R]	5-8	7
Immustart[R]	6-15	9
HEAD*START*[R]	10-27	16
Lifeline[R]	5-10	7

Table 6 Apparent absorption of the consumed immunoglobulins in calves fed 2 doses of colostrum supplements

Colostrum Source	Mass of Ig consumed (g)	Mean Serum Ig (g/L)	Apparent Effective Absorption* (%)
Colostrx[R]	74	7	30
Immustart[R]	88	9	32
HEAD*START*[R]	120	16	42
Lifeline[R]	114	7	22

*** % Apparent absorption is calculated as follows: average serum immunoglobulins (g/L) x 7% of body weight (kgms)/mass of Ig consumed (gms).

These data show that very high serum immunoglobulin levels were attained in calves fed 2 litres of maternal colostrum, calves fed HEAD*START*[R] achieved high serum IgG levels while those fed other supplements attained levels lower than is generally considered adequate for normal neonatal health (levels > 10 g/L).

The proportion of the fed mass of immunoglobulins absorbed was highest for the colostrum-derived product with high % immunoglobulin (HEAD*START*[R]) and significantly lower for Immustart[R] and Colostrx[R] which are colostrum and whey derived (respectively) but with lower % immunoglobulin. The percentage absorbed was very low for the serum-derived product, Lifeline[R] in spite of the relatively high immunoglobulin mass in this product.

Taken together the results of these studies have shown that to use colostrum supplements to achieve normal protective levels of serum immunoglobulins in neonatal calves the mass of the immunoglobulin fed and their % absorption must be in excess of that which is achieved with 3/4 supplements tested. With 3/4 supplements the % immunoglobulin is less than 25% which may contribute to lower rates of absorption than in the product with higher % immunoglobulin. It was shown experimentally that adding excess whey protein to the supplement diminished the % immunogloblin absorbed. Colostral trypsin inhibitor was not affected by freezing or spray drying and was high in 3/4 products tested. Low trypsin inhibitor levels were found in one product and may also contribute to diminished absorption in that product, however the product with the lowest absorption rate had the highest trypsin inhibitor level suggesting that this may not be the most important factor determining immunoglbulin absorption in neonatal calves. Overall, the most apparent problems with 3/4 colostrum supplements tested was suboptimal immunoglobulin mass coupled with low percentage immunoglobulin.

Personnel

One technical personnel, Mr R Sargent has been partially supported by this grant . Calf feeding has been performed on contract basis by Ms L Jensen, Osler, SK.

Equipment

No equipment has been purchased with the funds received for this study.

Project Developed Materials

None currently available

Project Photos

None currently available

Acknowledgment

The results of these studies will be prepared for publication in the Canadian Veterinary Journal, the SDAF support will be acknowledged.

Expense Statement

Copy of summary of expenses statement has been sent directly from the University of Saskatchewan Office of Research Services.

Appendix 1: Radial Immunodiffusion (RID) Assay for the detection of IgG.

1. Prepare 1.0% Seakem ME Agarose by dissolving 0.15 g agarose in 15.0 mL PBS (pH = 7.25) in a 50 mL Falcon tube. Place the screw cap on loosely and bring to a boil in the microwave oven (full power for 55 seconds).
2. Cool the agarose solution to 56 deg C using a water bath.
3. Dispense 13.8 ± 0.1 mL of agarose into 16 x 100 mm culture tubes. Add 10 µL of 10% sodium azide (10 gms of NaN3 in 100 mLs of POUR water).
4. Add 1.20 mL of the rabbit anti-immunoglobulin G into each tube and gently mix to ensure a homogeneous mixture without bubbles.
5. Select an empty immunodiffusion plate and place it on a levelled table. Immediately pour the melted agar mixture (15 mL) into the plate. Allow the agar to harden at room temperature (20-25 deg C) for 15 minutes.
6. Place the plate in a humidified chamber.
7. Using a punch (2.0 mm) and an RID template, cut wells in the agar plate. Each well should be 2.0 mm diameter (about 6 µL volume) and separated from its neighbours by 13 mm.
7. Prepare dilutions of the Reference Ig Standard (Bovine Immunoglobulin G) to obtain immunoglobulin concentrations of 0.375, 0.75, 1.5, 3, and 6 mg/mL using PBS (pH 7.25). These plates have a maximum IgG range of 0.375-6.0 mg/mL. The Ig concentration(s) of the original Stock Reference Ig Standard was evaluated by Bethyl Laboratories as described in Appendix 3.
9. Prepare dilutions of the following reagents in PBS (pH 7.25):
    Control Bovine Serum (diluted 1/10)
    Control Colostrum (Serial # 130) (diluted 1:40)
10. Prepare a Stock Solution of the Test Serial (225 g of the product plus 750 mL POUR water to make a total of 1 L).
11. Prepare a 1/40 dilution of the Test Serial.
12. Dispense 2 replicates of 4 µL of the diluted Standard Serial on the plate.
13. Dispense 4 µL of each dilution of the Ig reference serum.
14. Dispense 2 replicates of 4 µL of each dilutions of the Control Serum on the plate.
15. Dispense 5 replicates of 4 µL of the diluted Test Serial on the plate.
16. Incubate all plates at room temperature (20-25 deg C) for 24 hours in a humid chamber.
21. Using the plate reader, examine the plates at 24 hour incubation; record the Ring Diameters for the precipitin rings.
22. Using the results (ring diameters) obtained for each of the concentrations of the Reference Ig Standards generate a regression line (for each plate) for the variables R (ring diameter) vrs log10 (conc).
23. The plate is acceptable if the R2 is ≥ 0.98 and the values obtained for the Standard Serial and Standard Serum are the expected values ± 10%.
24. Ig concentration for the Test Serial is determined using the Regression line (for the Reference Ig Standard) obtained for each plate and the average of the observed (measured) ring diameters for the Test Serial.
25. The values obtained for the replicate tests of the Test Serial are acceptable if at least 4/5 values are less than ± 10% the mean of the five values, if the differences among 2/5 of the replicates (or more) is ≥ 10%, the test is repeated.

Appendix 2. Assay for determination of levels of trypsin inhibitor in bovine colostrum, milk and serum.

The assay for determination of levels of trypsin inhibitor activity is a radial protease diffusion method designed to measure the inhibition of trypsin-induced proteolysis of milk casein.

1. Diffusion plates were prepared using 5.0 % skim milk powder (Difco Laboratories, Detroit, MI) in phosphate buffered saline (0.01 M PBS, pH= 7.4). Agarose was reconstituted in the 5% skim milk/PBS to yield a 2% suspension.

2. The agarose/milk mixture was poured into immunodiffusion plates (8.4 cm x 9.4 cm, Pharmacia) and cooled to form a solid gel. Wells were punched in the gel using a 2.5 mm punch and the gel plug aspirated with a pipette attached to a suction apparatus.

3. A stock trypsin solution of 30 mgm/mL was prepared in PBS. The stock solution was then diluted (1/150 (vol/vol) to 0.2 mg/mL.

4. All samples were warmed to 37 degrees C prior to testing.

5. Samples were diluted 1/4 (vol/vol) in PBS (Gibco BRL). Equal volumes (15 mL) of diluted samples and trypsin solution were mixed and incubated for 15 minutes at 22 degrees C, then 4 µL of each mixture and a series of dilutions of the unadulterated trypsin solution were added to duplicate wells of the plate. The plates were incubated for 22 hours at 22 degrees C in a humid chamber.

6. The diameters of the rings of casein degradation were apparent as clear zones in the cloudy gel and were measured with a radial immunodiffusion plate reader (Transdyne General Corp. 2743).

7. The ring diameters obtained with the trypsin solution/sample mixtures were subtracted from those obtained with the trypsin solution alone (at 100 µg/mL) to determine the amount of protease inhibition. Results are expressed as the mg of trypsin inhibited/mL of sample. The results reported are the average values obtained with duplicate samples tested on two plates. The plate to plate variability was $\leq$ +/- 5% for both the noninhibited trypsin solution and test samples.